



Integrin 1, osmosensing, and chemoresistance in mouse ehrlich carcinoma cells

Sørensen, Belinda Halling; Rasmussen, Line Jee Hartmann; Broberg, Bjørn Sindballe; Klausen, Thomas Kjær; Sauter, Daniel Rafael Peter; Lambert, Ian Henry; Aspberg, Anders; Hoffmann, Else Kay

Published in:
Cellular Physiology and Biochemistry

DOI:
[10.1159/000374057](https://doi.org/10.1159/000374057)

Publication date:
2015

Document version
Publisher's PDF, also known as Version of record

Document license:
[CC BY-NC](#)

Citation for published version (APA):
Sørensen, B. H., Rasmussen, L. J. H., Broberg, B. S., Klausen, T. K., Sauter, D. R. P., Lambert, I. H., Aspberg, A., & Hoffmann, E. K. (2015). Integrin 1, osmosensing, and chemoresistance in mouse ehrlich carcinoma cells. *Cellular Physiology and Biochemistry*, 36(1), 111-132. <https://doi.org/10.1159/000374057>

Original Paper

Integrin β_1 , Osmosensing, and Chemoresistance in Mouse Ehrlich Carcinoma Cells

Belinda Halling Sørensen Line Jee Hartmann Rasmussen Bjørn Sindballe Broberg
Thomas Kjær Klausen Daniel Peter Rafael Sauter Ian Henry Lambert
Anders Asperg Else Kay Hoffmann

Department of Biology, Section of Cell and Developmental Biology, The August Krogh Building,
University of Copenhagen, Copenhagen Ø, Denmark

Key Words

Integrin • Cisplatin • Gemcitabine • Ehrlich Ascites Tumor Cells • Multi-drug-resistance • Hypotonic cell swelling

Abstract

Background/Aims: Altered expression of the integrin family of cell adhesion receptors has been associated with initiation, progression, and metastasis of solid tumors as well as in the development of chemoresistance. Here, we investigated the role of integrins, in particular integrin β_1 , in cell volume regulation and drug-induced apoptosis in adherent and non-adherent Ehrlich ascites cell lines. **Methods:** Adhesion phenotypes were verified by colorimetric cell-adhesion-assay. Quantitative real-time PCR and western blot were used to compare expression levels of integrin subunits. Small interfering RNA was used to silence integrin β_1 expression. Regulatory volume decrease (RVD) after cell swelling was studied with calcein-fluorescence-self-quenching and Coulter counter analysis. Taurine efflux was estimated with tracer technique. Caspase assay was used to determine apoptosis. **Results:** We show that adherent cells have stronger fibronectin binding and a significantly increased expression of integrin α_5 , α_v and β_1 at mRNA and protein level, compared to non-adherent cells. Knockdown of integrin β_1 reduced RVD of the adherent but not of the non-adherent cells. Efflux of taurine was unaffected. In contrast to non-adherent, adherent cells exhibited chemoresistance to chemotherapeutic drugs (cisplatin and gemcitabine). However, knockdown of integrin β_1 promoted cisplatin-induced caspase activity in adherent cells. **Conclusion:** Our data identifies integrin β_1 as a part of the osmosensing machinery and regulator of cisplatin resistance in adherent Ehrlich cells.

Copyright © 2015 S. Karger AG, Basel

B.H. Sørensen and L.J.H. Rasmussen contributed equally to this work.

Else Kay Hoffmann, Professor,

Department of Biology, The August Krogh Building, Universitetsparken 13, DK-2100,
Copenhagen Ø, (Denmark)
E-Mail ekhoffmann@bio.ku.dk

Introduction

Integrins play a crucial role in the regulation of several biological processes implicated in tumor progression, including cell migration, invasion, proliferation, and tumor cell survival [1-3]. Integrins are a family of enzymatically inactive cell adhesion receptors, comprising 18 different α subunits and eight different β subunits that assemble non-covalently as membrane-spanning heterodimers, forming at least 24 different integrins with distinct ligand binding specificities. Integrin β_1 associates with 12 different α subunits and thereby constitutes the largest subgroup of integrin receptors [1, 4].

Integrins are adhesion molecules that mediate cell-cell interactions and attachment of the cell to various extracellular matrix (ECM) components, e.g., laminin, collagen, and fibronectin [1, 4]. Integrin binding to ECM ligands leads to integrin clustering and formation of focal adhesions and induces intracellular signaling, mainly through the engagement of various signaling proteins associated with the focal adhesions, such as the focal adhesion kinase (FAK) and the cytosolic tyrosine kinase Src. The ability of integrins to regulate important cellular processes is due to the activation of these downstream signaling molecules, leading to activation of the phosphatidylinositol 3'-kinase (PI3K), the serine/threonine kinase Akt, as well as the extracellular regulated kinase (ERK)-cascade [5]. It is well known that loss of adhesion results in cells undergoing apoptosis (i.e. anoikis) [6].

The ability to maintain and regulate cell volume is a fundamental requirement for mammalian cells to carry out normal cell functions, including proliferation, migration, and apoptosis [6-9]. Cells respond to volume perturbations by activating volume regulatory systems within the cells [9]. The processes by which a swollen or shrunken cell returns to its steady-state volume are known as regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively. In Ehrlich cells, RVD is carried out by decreasing the Na^+ permeability and greatly increasing the permeability towards K^+ , Cl^- , and organic osmolytes, such as taurine [9, 10].

Although much is known about cell volume regulation, the mechanisms of volume sensing in mammalian cells are still incompletely understood. In bacteria, archaea, fungi, and plants the volume sensor consists of a two-component histidine kinase system, resulting in the activation of the high osmolarity glycerol 1-mitogen activated protein (HOG1-MAP) kinase [9, 11, 12]. Although MAP kinases exist in mammalian cells, the histidine kinase system is lacking, and volume sensing must therefore rely on other undiscovered osmosensory mechanisms [9].

A number of cellular components have been speculated to be the volume sensor, including receptors and cell adhesion proteins (e.g., integrins, growth factor (GF) receptors, cytokine receptor tyrosine kinases (RTKs), and calcium-sensing receptors), stretch-activated channels, transient receptor potential receptors (TRPs), phospholipases (PLA_2 and PLC), lipid kinases (PI3K and PI5K), the cytoskeleton (e.g. actin), and small GTP-binding proteins (e.g. Rho, Rac, and Cdc42), as reviewed in [13].

In hepatocytes, the integrin system has been identified as the major osmosensory system activated upon hypotonic cell swelling, as hypotonic perfusion of rat liver led to a rapid appearance of the active conformation of integrin β_1 [14]. Furthermore, hypotonic hepatocyte swelling was followed by an Arg-Gly-Asp (RGD)-peptide sensitive activation of FAK and Src and integrin β_1 -dependent transactivation of epidermal growth factor receptor (EGFR), which triggered a signaling cascade involving activation of MAP kinases (i.e., ERK1/2 and p38^{MAPK}) and a volume regulatory K^+ efflux [15, 16]. Several volume-sensitive K^+ channels are found to be regulated by integrin β_1 including $\text{K}_{\text{v}}1.3$ [17-19] and $\text{K}_{\text{v}}11.1$ [20]. Artym and Petty have by two-step fluorescence resonance energy transfer (FRET) provided evidence for a physical linkage between β_1 integrins and $\text{K}_{\text{v}}1.3$ in an adherent human melanoma cell line LOX, but not in LOX cells cultured in suspension, indicating that the adherent phenotype is important for the interaction [17].

Browe and Baumgarten investigated the role of integrin activation on the swelling-induced activation of the volume regulated Cl^- channel (VRAC) in cardiac myocytes and

found that integrin stretch was associated with an activation of FAK, Src, and the angiotensin II receptor, leading to EGF receptor transactivation and resulted in the activation of PI3K, Rac, and H_2O_2 production [21-24]. H_2O_2 leads to the activation of a Cl^- current very similar to that mediated by VRAC in response to hypotonicity [22, 23]. In Ehrlich cells, hypotonic cell swelling is accompanied by an increased production of reactive oxygen species (ROS) which stimulates KCl co-transport in non-adherent EATC-WT cells, but separate K^+ , Cl^- , and taurine efflux channels in adherent ELA cells [25, 26].

Resistance against chemotherapeutic agents is one of the major challenges in cancer treatment and accounts for the majority of treatment failures in cancer patients. Drug resistance can either be an innate property (intrinsic resistance) due to rare genetic changes or be acquired during chemotherapy (extrinsic resistance) [27]. Several *in vitro* studies have implicated the integrin β_1 subunit in the development of resistance towards different chemotherapeutic compounds in various cancers, including solid tumors of the breast [28-32], bladder [33], prostate [34, 35], lungs (small cell and non-small cell lung cancer) [36-38], and pancreas [39, 40], as well as hematological malignancies, such as multiple myeloma, myeloid and B lymphoid malignancies [41, 42]. In addition, clinical studies have shown an association between increased integrin β_1 expression and decreased survival and poor prognosis in patients with invasive breast cancer and small cell lung carcinoma [43, 44]. Furthermore, inhibition of integrin β_1 reduces chemoresistance in bladder cancer [33] and enhances the sensitivity to radiation treatment in human breast cancer xenografts [45]. The integrin β_1 -mediated tumor cell survival and chemoresistance appear to be facilitated by adhesion to integrin β_1 ligands, including fibronectin, collagens, and laminin, causing the activation of downstream signaling molecules, where PI3K and Akt play major roles [5].

In this study, we investigate the role of adhesion in cell volume regulation, survival, and chemoresistance, using a model system comprising the three murine Ehrlich cell lines; Ehrlich Ascites Tumor Cell-wild type (EATC-WT), EATC-Multidrug resistant (EATC-MDR), and Ehrlich Lettré Ascites (ELA) cells. These three cell lines differ on the basis of their different adhesive and resistance properties [46-48]. Our studies show that adherent ELA cells have a significantly higher expression of the integrin subunits integrin α_5 , α_v , and β_1 compared to the two related suspension cell lines (EATC-WT and EATC-MDR). We also find that integrin β_1 has a function in volume sensing and drug resistance in ELA cells but not in the suspension cells EATC-WT and EATC-MDR. Contribution of integrin in β_1 RVD and cisplatin resistance did neither involve regulation of VSOAC nor activation of FAK, respectively.

Materials and Methods

Solutions and reagents

Cisplatin was from Sigma-Aldrich and dissolved at 5 mM in ddH_2O . To avoid spontaneous *cis* to *trans* isomerisation, cisplatin aliquots were discarded after three freeze-thaw cycles. Gemcitabine (GEMZAR®, Eli Lilly) was dissolved at 50 $\mu g/\mu l$ in phosphate buffered saline (PBS). "Glycine-Arginine-Glycine-Aspartate-Serine" (GRGDS) peptide was from Sigma and dissolved in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma). Ethylenediaminetetraacetic acid (EDTA) was from ChemSolute (Th Geyer) and dissolved in ddH_2O (pH 8.0). The FAK inhibitor PF-573,228 (PF; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM and stored at $-80^\circ C$. The final concentration used on the cells was 10 μM .

Primary antibodies against integrin α_5 (AB1928), α_v (AB1930), and β_1 (AB1952) were from Millipore and were all used in a dilution of 1:2000. HSC70 (sc-1059) antibody (1:1000) was from Santa Cruz Biotechnology (Dallas, Texas), and β -actin antibody (1:1000) was from Cell Signaling (Danvers, MA). Alkaline phosphatase- and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Sigma (St. Louis, MO) and Dako (Glostrup, Denmark), respectively.

Cell lines and cell culturing

We used three closely related murine cell lines; Ehrlich Lettré Ascites (ELA), Ehrlich Ascites Tumor cells-wild type (EATC-WT), and EATC-multidrug resistant (EATC-MDR). The EATC-WT cells originate from

spontaneously arisen mammary gland adenocarcinomas in aged female mice. The tumors were transplanted to other mice, and cells were cultured from the transplanted tumors, giving rise to the cell lines used in this study [49, 50]. EATC-MDR was developed as a multidrug-resistant substrain from an originally drug-sensitive strain of EATC-WTs by subjecting them to daunorubicin treatment for several passages. The cells show cross-resistance towards the antibiotic adriamycin and the vinca alkaloids vinblastine and vincristine [51]. Both EATC-WT and EATC-MDR are non-adherent, suspension cells. The ELA cells are an adherent subtype of EATC-WT that grows as a monolayer, thereby having closer resemblance to the epithelial tissue the tumor originated from [46].

ELA, EATC-WT, and EATC-MDR were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma), at 37°C, 5% CO₂, and 95% humidity. Cultures were passaged every 3-4 days, and only passages 5-30 were used. To maintain multidrug resistance, EATC-MDR cells were selected every 21 days with 42 μ M daunorubicin (Sigma-Aldrich) for four days. Cells were not used for experiments until they had had at least had one daunorubicin-free passage.

Small interfering RNA-mediated knockdown of integrin β_1

Transient transfection of the cells was mediated with annealed, 21-bp small interfering RNA (siRNA) duplexes targeting murine integrin β_1 . The siRNA was pre-designed and obtained from Ambion (Life Technologies, siRNA ID # s68454), and the sequence for the antisense strand was 5'-CAACUGUGAUAGGUCUAAUtt-3', targeting exon 12 in mouse integrin β_1 . A 21-bp negative control oligomer (Silencer Select No. 1 siRNA, Ambion) was used as a negative control and to create a baseline for transfection efficiency. Cells were transfected with integrin β_1 - or negative control (Mock) siRNA at 5 nM with DharmaFECT 1 Transfection Reagent (Thermo Scientific). After 24 h, the medium was replaced by transfection reagent-free medium, and the cells were used in experiments as indicated. Knockdown was confirmed by western blot analysis of integrin β_1 protein level.

Cell adhesion assay

NUNC MaxiSorp 96-well microtiter plates (Thermo Scientific) were coated with bovine plasma fibronectin (Invitrogen) in PBS (136.89 mM NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄, 1.47 mM KH₂PO₄) supplemented with Ca²⁺ and Mg²⁺ (1 mM CaCl₂ and 0.5 mM MgCl₂, pH 7.4) and incubated in a humid chamber over night at 4°C. The coating solution was removed, the wells were washed three times with PBS, followed by 1-2 h blocking with PBS containing 1 mg/ml bovine serum albumin (BSA; Sigma). Next, the wells were washed once with PBS, and 100 μ l RPMI-1640 medium supplemented with 2 mg/ml BSA were added to each well. The cells were washed, trypsinized with trypsin/EDTA, and washed with PBS supplemented with 0.5 mg/ml Soybean Trypsin Inhibitor (Invitrogen). Cells were resuspended in serum-free RPMI, counted using a modified Neubauer chamber, diluted with serum-free RPMI to a density of 250,000 viable cells/ml, and 100 μ l cell suspension were added to each well. The plates were incubated for 1 h at 37°C in presence or absence of adhesion modulators (i.e., EDTA or GRGDS), followed by three washes with PBS supplemented with Ca²⁺ and Mg²⁺ to remove unbound cells. Cells were fixed with 5% glutaraldehyde solution (Sigma) for 20 min, permeabilized with 20% methanol for 10 min, and subsequently stained with Crystal Violet (0.5% in 20% methanol; Sigma) for 20 min. The plates were washed thoroughly with water, and the dye dissolved with 10% acetic acid. Absorbance was read at 570 nm (Multiskan ascent, Thermo Scientific) to quantify the amount of crystal violet taken up by the cells. To translate crystal violet absorbance into numbers of cells, a standard curve was made for each cell line in a separate uncoated, unblocked plate, by seeding 0, 5 \times 10³, 12.5 \times 10³, and 25 \times 10³ cells per well in triplicates in 100 μ l serum-free RPMI. The standard curve plate was not washed before glutaraldehyde fixation.

Real-time quantitative polymerase chain reaction (qPCR)

Cells were grown to 80% confluency, washed with PBS, and total RNA was isolated and purified with the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instruction. Purity and concentrations were determined using NanoDrop spectrophotometry and agarose gel electrophoresis. RNA was reverse transcribed using Omniscript Reverse Transcription kit (QIAGEN), according to the manufacturer's instruction. Quantitative PCR was performed in triplicates for each cDNA sample, using 1 μ l cDNA and 19 μ l qPCR MasterMix containing EXPRESS SYBR® GreenER™ SuperMix Universal (Invitrogen), ddH₂O, and 0.2

μ M of one of the following primer pairs: Integrin α_2 : Fw: 5'-TGA CAG CAG CGG CAG AGA TCG A-3', Rv: 5'-AAG CCG AGC TTC CAC AAA CCT GC-3'; Integrin α_3 : Fw: 5'-AGC TGT CCA CGT CGA GTC ACC A-3', Rv: 5'-AGC CTC ACC CAT CAC TGT GCC A-3'; Integrin α_5 : Fw: 5'-GCA GGA TAG AGG ACA AGG CCC AGA-3', Rv: 5'-AAG CTC GGC TTC ATA GGC ACC G-3'; Integrin α_6 : Fw: 5'-ACC TCG GCA CAG CAA CCT TGA-3', Rv: 5'-ACG CTG CAG TTG AGA GTC TGG T-3'; Integrin α_v : Fw: 5'-TGT CCG GAA CAA CGA AGC CTT AGC-3', Rv: 5'-TGA CTG CTG GTG CAC GCT GAA-3'; Integrin β_1 : Fw: 5'-TTG TGG AGG CAA TGG CGT GTG C-3', Rv: 5'-ACA GAC GCC AAG GCA GGT CTG A-3'; Cyclophilin A: Fw: 5'-CCA CCG TGT TCT TCG ACA T-3', Rv: 5'-CAG TGC TCA GAG CTC GAA AG-3'. qPCR was performed using a Bio-Rad CFX96 machine (2 min at 50°C, 10 min at 95°C, 40 cycles of 30 seconds at 95°C, 1 min at 60°C, 30 seconds at 72°C). The program was terminated and dissociation curves determined by 1 min at 95°C, 30 seconds at 55°C, and a terminal temperature increase to 95°C (1°C/min). Data were analyzed with the Bio-Rad CFX manager 1.5 software (BioRad). The efficiency for each primer pair reaction was calculated by the software based on a standard curve. Data were normalized using cyclophilin A as a reference gene and set relative to the adherent ELA cells, thus giving a $\Delta\Delta C_T$ -value.

SDS-PAGE and western blotting

Adherent cells were grown to 80% confluency in 21 cm² petri dishes, while 2 million suspension cells were grown in 50 ml suspension culture flasks at the beginning of each experiment. Cells were washed in ice-cold PBS and lysed in 100 μ l 95°C SDS lysis buffer (1% SDS, 1 mM Na₃VO₄, 10 mM Tris HCl, pH 7.4) supplemented with cOmplete Mini protease inhibitor cocktail (Roche Applied Science). Lysates were homogenized by sonication, followed by removal of cell debris by centrifugation at 20,000 *g* for 5 min. Protein concentration of cleared total cell lysates was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) and BSA as the protein standard. Samples for electrophoresis were prepared with NuPAGE LDS Sample Buffer (Novex by Life Technologies), dithiothreitol (DTT), ddH₂O, and equal amount of protein. *Note:* Samples used for integrin α -subunit-detection were prepared without DTT. Samples were boiled for 5 min and equal amounts of protein (30 μ g) were loaded to each well. SDS-PAGE gel electrophoresis was carried out in Novex chambers with NuPAGE precast Tris-Acetate 7% gels or Bis-Tris 10% gels under denaturing and reducing conditions using a BenchMark Protein Ladder (Novex). Separated proteins were electrotransferred to nitrocellulose membranes. Membranes were stained reversibly with Ponceau S Solution (Sigma-Aldrich) to confirm equal loading. The membranes were blocked (5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1 % Tween 20)) for 1 h at 37°C, incubated with primary antibodies diluted in blocking buffer overnight at 4°C, washed extensively in TBST, and incubated for 1 h with alkaline phosphatase- (1:3000) or HRP-conjugated (1:20,000) secondary antibody in blocking buffer at room temperature. Membranes were washed extensively in TBST and developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT; KPL) or Lumina Forte Western HRP Substrate (Millipore). Membranes probed with BCIP/NBT were scanned and protein bands quantified using UN-SCAN-IT gel 6.1 software (Silk Scientific). Chemiluminescence signal was captured using the ChemiDoc MP Imaging System and the Image Lab 4.0 software (Bio-Rad Laboratories).

Coulter counter experiments

Changes in EATC-WT cell volume upon hypotonic exposure were measured by electric cell sizing. Cells were grown to a density of 10⁶ cells/ml and carefully re-suspended. After 30-60 min incubation at 37°C, 5% CO₂, and 95% humidity, a 6 ml aliquot was taken and transferred to an Erlenmeyer flask placed in a shaking, pre-warmed (37°C) water bath. Absolute cell volumes were estimated after 50-fold dilution in either isotonic sucrose saline solution or hypotonic saline solution in a Beckmann Multisizer III (Coulter, Luton, UK), using an aperture size of 100 μ m calibrated with 20.47 μ m diameter latex beads (Coulter). All saline solutions were micro-filtered (Millipore filters, 0.45 μ m) before the experiments. The hypotonic saline solution (pH 7.4) used for coulter counter experiments contained: 71 mM NaCl, 2.5 mM KCl, 0.5 mM MgSO₄, 0.5 mM Na₃PO₄, 0.5 mM CaCl₂, 3.3 mM MOPS, 3.3 mM TES, and 5 mM HEPES. The isotonic sucrose saline solution with low ionic strength was obtained by adjusting the osmolarity of the hypotonic saline solution to 274 mOsm/kg H₂O with sucrose (approx. 40 g/L). Absolute cell volumes in fl (i.e., 10⁻¹⁵ L) were calculated from the mean cell diameter distribution curves obtained by the software. Results were represented as the relative cell volume as function of time, and the maximal recovery rate (fl/min) was calculated as the steepest numeric 1 min slope after reaching maximal cell swelling.

Calcein fluorescence self-quenching

Studies of RVD in ELA cells were performed by fluorescence self-quenching with calcein as a marker. Cells were grown to 70–80% confluency on pre-cleaned 15 mm glass coverslips placed in petri dishes. The coverslip was washed 3 times in 2 ml pre-warmed (37°C) isotonic saline solution (143 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM Na_3PO_4 , 1 mM CaCl_2 , 3.3 mM MOPS, 3.3 mM TES, 5 mM HEPES; pH 7.4). Cells were incubated for 10 min in isotonic saline solution with 5 μM CellTrace calcein Red-Orange-AM (Invitrogen), washed twice in pre-warmed isotonic saline solution (37°C), and the coverslip was mounted in a RC25 diamond bath perfusion chamber (Warner Instruments). The chamber was installed on an Imic2000 inverted fluorescence microscope (TILL Photonics), and cells were continuously superfused with saline solution heated to 37°C, using an inline perfusion heater (SH27B with TC324B controller, Warner Instruments). Cells were illuminated through a 40X/0.55 NA Objective (Nikon Plan40) at 555 ± 2.5 nm for 80 ms, using a Polychrome V monochromator (TILL Photonics). Illumination and fluorescence light was filtered through a red light specific filter set (Cat. 31002a, Chroma Technology), and images were digitized using an iXon 895 EMCCD camera (Andor Technology) with 2X binning and recorded using the Live Acquisition software package (TILL photonics). The cells were superfused with isotonic saline solution for the first 3 min and then switched to hypotonic saline solution for the next 10 min. Cells remaining in the petri dish following removal of the coverslip were washed twice in ice-cold PBS, lysed in RIPA buffer (1% Nonidet-P40, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1% Sodium deoxycholate, 1 mM Na_3VO_4 , and cComplete proteinase inhibitors) and stored (-20°C) for later analysis of knockdown efficiency. Single cell fluorescence was analyzed using the Offline analysis software (TILL Photonics). Regions of interest were drawn around single cells, and fluorescence intensity was quantified for individual cells throughout the experiment. Due to calcein leakage and fluorescence bleaching, the signal suffered from significant rundown, fitting a mono exponential decay. Before further calculations were performed, all signals were rundown corrected. The results are shown as the reciprocal relative fluorescence intensity (FI) as function of time, and the maximal recovery rate (FI/min) was calculated as the steepest numeric slope of the RVD response.

Estimation of taurine efflux by tracer technique

Swelling-induced taurine efflux in ELA cells was estimated by tracer technique as previously described [26]. Control, mock-, and integrin β_1 -siRNA transfected ELA cells were grown to 80% confluency in 6-well polyethylene culture plates and loaded with ^3H -taurine (37,000 Bq/well, PerkinElmer) in complete growth medium for at least 2 h (37°C, 5% CO_2 , 100% humidity). At the beginning of the experiment, each well was washed 3 times with 1 ml isotonic NaCl medium (300 mOsm; 143 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES; pH 7.4) per well to remove remaining extracellular isotope and growth medium. The experiment was performed at room temperature by transferring the NaCl medium from each well to individual scintillation vials (Snaptwist Scintillation vial, 6.5 ml, VWR) and replacing it with 1 ml new medium. This procedure was repeated at 2 min intervals for a total of 30 min. After the first 8 min, the isotonic medium was replaced by hypotonic medium (200 mOsm; 92 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES; pH 7.4). At the end of the experiment, remaining intracellular isotope was extracted by addition of 1 ml 1 M NaOH (1 h, shaking table) followed by two additional washes with ddH_2O . NaOH lysates and water washouts were transferred to individual vials. All vials were added 3.5 ml scintillation fluid (Packard™ Ultima Gold, PerkinElmer), mixed carefully and proceeded for β -scintillation counting in a Perkin Elmer scintillation counter (Waltham, MA). The total amount of ^3H -labeled taurine taken up by the cells in each well was calculated as the sum of ^3H activity released during the efflux experiment and in the NaOH/water washouts. The fractional rate constant (k , min^{-1}) for taurine release was calculated from the following equation: $k = [\ln(X_1) - \ln(X_2)] / (t_1 - t_2)$, where X_1 and X_2 are the fraction remaining in the cell at time t_1 and t_2 .

Caspases activity

The Homogeneous Caspases Assay (Roche Applied Science) was used for quantitative *in vitro* determination of caspase activity according to the manufacturer's instructions. Briefly, cells were seeded in black 96-well plates, and apoptosis was induced. After 24 h incubation at 37°C, the plates were frozen at -80°C for at least 30 min to lyse the cells. Substrate (DEVD-R110) Stock Solution was diluted 1:10 in Incubation Buffer, and 100 μl substrate solution were applied to each well. The plate was incubated at 37°C for 1 h protected from light, and finally, fluorescence was measured with a FLUOstar OPTIMA plate reader (Ramcon A/S). Caspase activity was normalized to an untreated control.

Cell viability assay – MTT

The colorimetric MTT assay was used to determine cell viability in ELA cells transiently transfected with integrin β_1 siRNA. ELA cells were counted and seeded to a density of 5,000 cells per 200 μ l media per well in 96-well plates and placed in the cell culture incubator (37°C, 5% CO₂, 100% humidity) for 72 h. At day two, cells were transfected with integrin β_1 or negative control siRNA. After 24 h, the media was changed to transfection-reagent-free media and the cells were left for another 24 h. At the end of the incubation period, 100 μ l was removed, 25 μ l of a MTT solution (5 mg/ml MTT in sterilized PBS) was added to each well, and the plate was incubated in the cell culture incubator for 3 h. Following incubation 100 μ l freshly made 10 mM HCl containing 1% SDS was added to each well and the plate incubated in fume hood overnight to solubilize the colored formazan crystals. Samples were measured at 540 nm using Wallac Envision Multilabel plate reader (Perkin Elmer). Data were represented relative to the untreated control cells. Each experiment was performed in quadruplicates.

Statistical analysis

Data are shown as mean with standard error of the mean (SEM). The number of independent experiments (n) is indicated for each experiment. Significance was evaluated using paired Student's *t*-test (two-tailed), one-way ANOVA, or two-way ANOVA with *P* < 0.05 indicating statistically significant difference.

Results

ELA cells exhibit strong fibronectin binding capacities

As adherence is the main phenotypic difference between ELA cells and the EATC (WT and MDR) cells, we characterized differences in their binding capacities to fibronectin. ELA, EATC-WT, and EATC-MDR cells were all able to bind to fibronectin, but the adherent ELA cells had a 2-fold higher binding capacity than the non-adherent cells (Fig. 1A and 1B). There was a clear dose-response in adherence for all three cell lines to an increasing coating concentration of fibronectin (Fig. 1A), however, ELA cells reached half maximum adhesion (EC₅₀) at a significantly lower concentration (0.5±0.2 μ g/ml) than EATC-WT (3.7±0.2 μ g/ml, *P*<0.001) or EATC-MDR (1.5±0.2 μ g/ml, *P*<0.001). Interestingly, the EC₅₀ of the EATC-MDR cells was markedly lower than EC₅₀ of EATC-WT, indicating that the multidrug resistant EATC-MDR cells possess better fibronectin binding capacities than the wild type cells. In

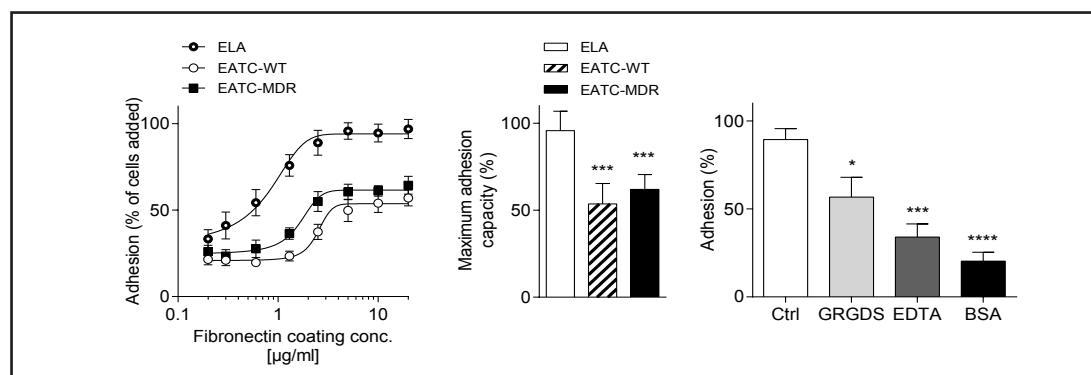


Fig. 1. Adhesion of ELA, EATC-WT, and EATC-MDR cells to fibronectin. ELA, EATC-WT, and EATC-MDR cells were seeded in 96-well plates coated with (A) increasing concentrations or (B) 2 μ g/ml of fibronectin supplemented with Ca²⁺ and Mg²⁺ and incubated for 1 h at 37°C. Subsequently, cells were fixed with glutaraldehyde, permeabilized with methanol, stained with Crystal Violet, and absorbance was measured at 570 nm. The number (n) of independent experiments was 4-5. A. Dose-response curves for adhesion to increasing concentrations of fibronectin. B. Maximum adhesion capacity to fibronectin. C. Inhibition of ELA cell adhesion by treatment with 5 mg/ml GRGDS peptide or 10 mM EDTA, compared to an untreated control. BSA indicates the background. Statistical analysis was performed using one-way ANOVA with Tukey-Kramer multiple comparisons post test, where *, ***, and **** indicate *P*<0.05, *P*<0.001, and *P*<0.0001, respectively.

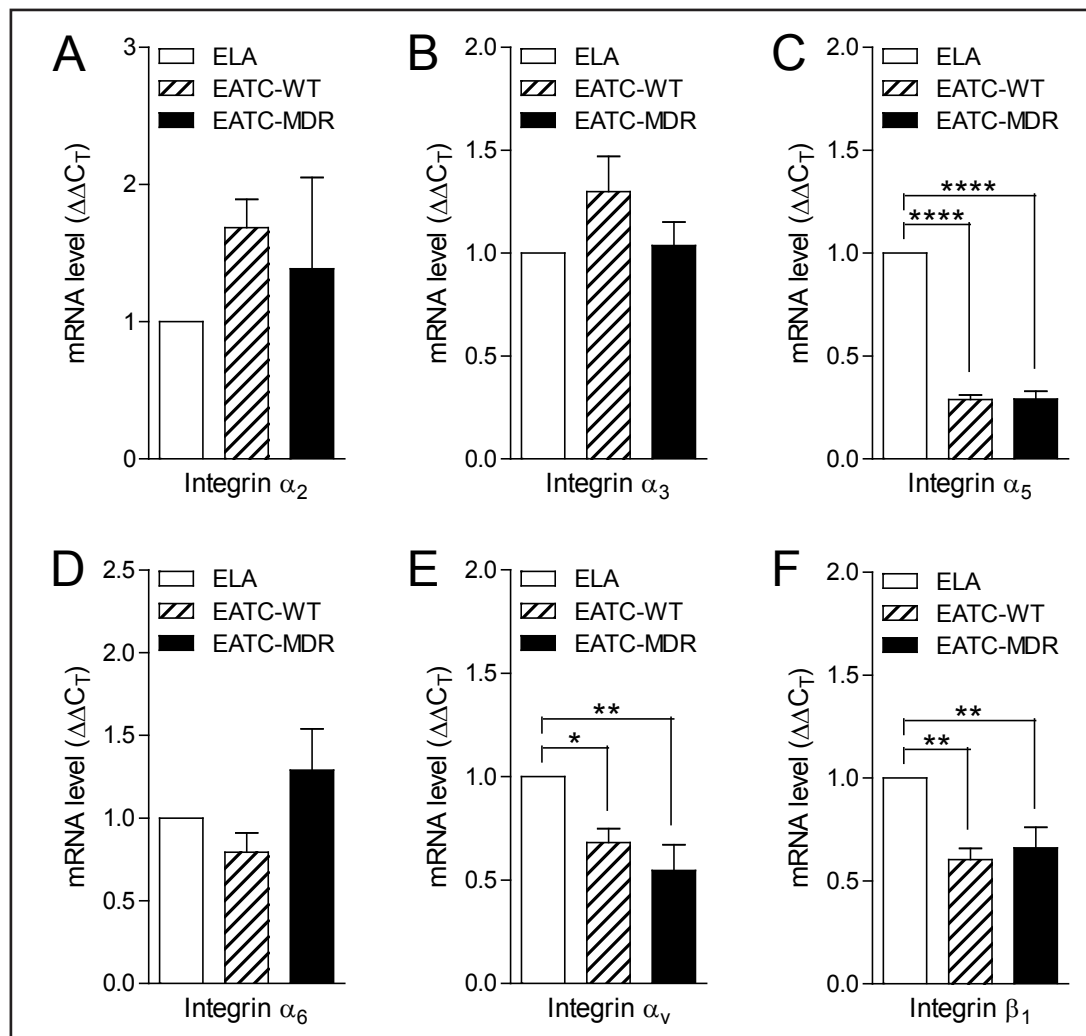


Fig. 2. Comparison of the mRNA expression levels of selected integrin subunits in ELA, EATC-WT, and EATC-MDR cells. Relative mRNA expression of integrin α_2 (A), α_3 (B), α_5 (C), α_6 (D), α_v (E), and β_1 (F) in ELA, EATC-WT, and EATC-MDR cells, determined by qPCR, using specific primer pairs for each integrin subunit and cyclophilin A as reference gene. Data was normalized against cyclophilin A and corresponding ELA sample. Statistical analysis was performed using one-way ANOVA with Tukey-Kramer multiple comparisons post test, where *, **, and **** indicate $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively. The number (n) of independent experiments was 4-5.

addition, we also found that adhesion of ELA cells to fibronectin was highly modulated by integrin inhibition through addition of the GRGDS peptide or the Ca^{2+} and Mg^{2+} -chelating agent EDTA (Fig. 1C). Seeding ELA cells on fibronectin (2 $\mu\text{g}/\text{ml}$ coating concentration) in the presence of 0.5 mg/ml GRGDS resulted in a 40% reduction in adhesion, and treatment with 10 mM EDTA was found to almost block the adhesion to fibronectin.

ELA cells have significantly higher mRNA and protein levels of integrin α_5 , α_v , and β_1

To compare the differences in integrin composition between the adherent ELA cells and the non-adherent EATC-WT and EATC-MDR cells, we next investigated the relative expression of a selection of integrin subunits using qPCR. These included integrins α_2 , α_3 , α_5 , α_6 , α_v , and β_1 .

By comparing the mRNA expression, we found that adherent ELA cells had significantly higher mRNA expression level of the fibronectin receptor subunits integrin α_5 and integrin β_1 (Fig. 2C and 2F), which was about 3.5-fold higher for integrin α_5 and 1.5-fold higher for

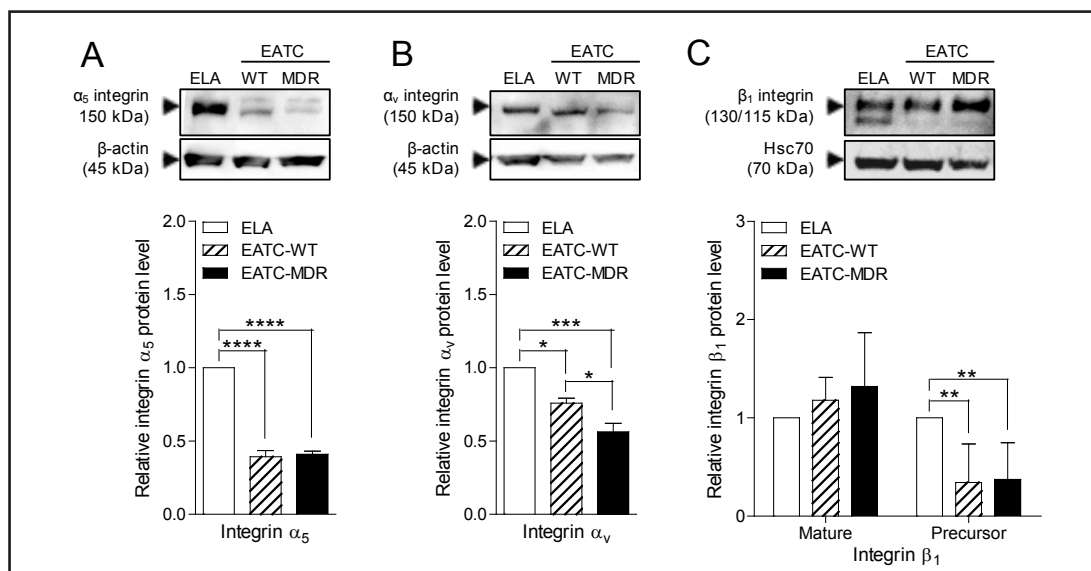


Fig. 3. Integrin α_5 , α_v , and β_1 protein levels in ELA, EATC-WT, and EATC-MDR cells. Western blot analysis of whole cell protein lysates of ELA, EATC-WT, and EATC-MDR cells. Protein expression of integrin α_5 (A), α_v (B), and β_1 (C) was determined using specific antibodies against integrin α_5 , α_v , and β_1 , respectively. Hsc70 or β -actin was used as loading controls. Data is shown as mean with SEM error bars relative to the expression in ELA cells. Statistical analysis was performed using one-way ANOVA with Tukey-Kramer multiple comparisons post test, where *, **, ***, and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively. The number (n) of independent experiments was 3-6.

integrin β_1 , than the two non-adherent cell lines EATC-WT and EATC-MDR. Similarly, there was a higher level of integrin α_v mRNA in the adherent ELA cells (Fig. 2E), which was 1.5- and 1.8-fold higher than EATC-WT and EATC-MDR, respectively. These data indicate that the adherent ELA cells have higher mRNA levels of the fibronectin receptor subunits, integrins α_5 , α_v , and β_1 , compared to the non-adherent EATC-WT and EATC-MDR cells. Neither the mRNA expression of collagen receptor integrin subunit α_2 nor of the two laminin receptor subunits integrins α_3 and α_6 were found to vary between any of the three Ehrlich cell lines (Fig. 2A, 2B, and 2D).

Next, we examined the protein level of the fibronectin receptor subunits, integrin α_5 , integrin α_v , and integrin β_1 , to see whether the differences observed on mRNA levels were correlated with the relative protein expression. Whole cell lysates of ELA, EATC-WT, and EATC-MDR cells were analyzed by western blot analysis, using specific primary antibodies for integrins α_5 , α_v , and β_1 . In support of our qPCR findings, the integrin α_5 protein level was found to be 2.5-fold higher in ELA cells relative to both EATC-WT and EATC-MDR (Fig. 3A). The integrin α_v protein level was 1.3- and 1.8-fold higher in ELA cells than EATC-WT and EATC-MDR (Fig. 3B), respectively, which also correlated with the relative mRNA expression.

Two bands were detected in the western blot analysis for integrin β_1 (Fig. 3C), presumably corresponding to the 130 kDa fully glycosylated mature form present on the cell surface, and the 115 kDa partially glycosylated integrin β_1 precursor [52]. Interestingly, western blot analysis revealed similar protein levels of the mature 130 kDa integrin β_1 isoform in all three cell lines, while the adherent ELA cells had a 3-fold higher expression of the immature 115 kDa integrin β_1 precursor compared to EATC-WT and EATC-MDR (Fig. 3C). Whether this difference represents higher turnover of integrin β_1 in ELA cells compared to the two suspension cell lines and/or merely reflects the higher expression observed with qPCR is unknown, and was not investigated further.

These western blot data support our qPCR findings with a significantly higher expression of the fibronectin receptor subunits integrins α_5 , α_v , and β_1 in ELA cells relative to the two non-adherent cell lines EATC-WT and EATC-MDR.

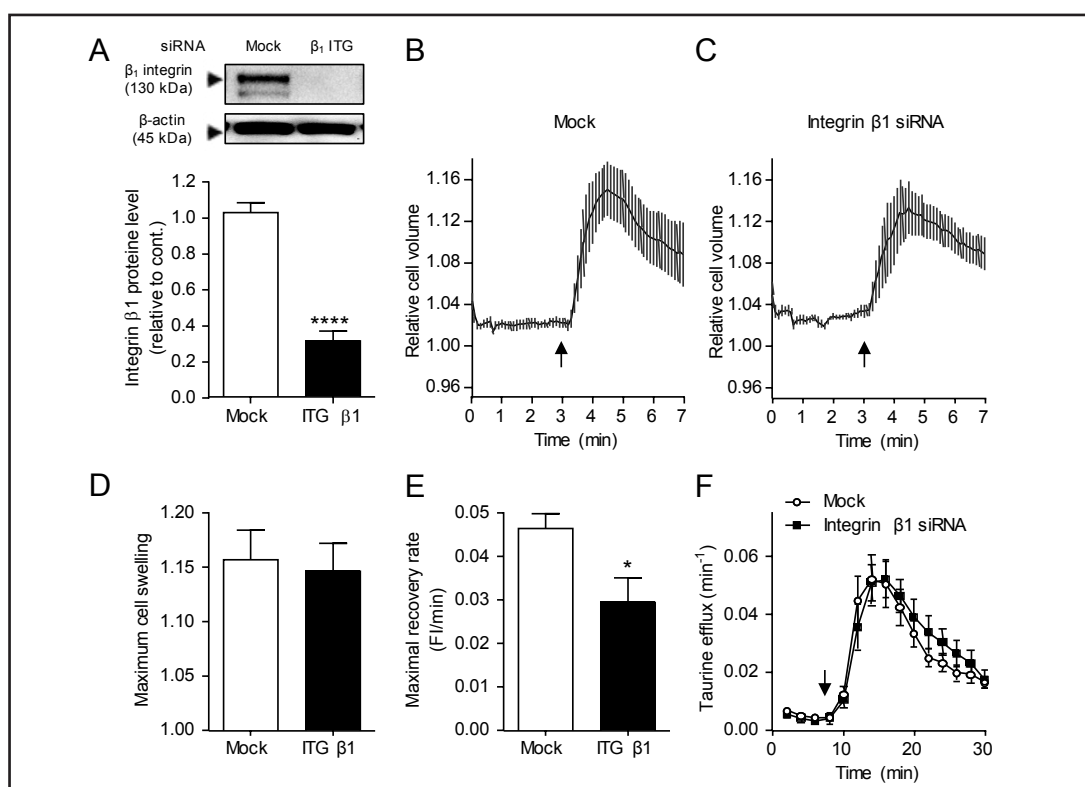


Fig. 4. Effect of siRNA-mediated knockdown of integrin β_1 on cell swelling and RVD in ELA cells. Changes in cell volume, RVD, and taurine efflux upon hypotonic exposure in ELA cells transfected with 5 nM mock- or integrin β_1 -siRNA for 24–48 h. A. Efficiency of siRNA knockdown (48 h) was determined with western blot analysis, using a specific anti-integrin β_1 antibody. β -actin was used as loading control. B and C. Changes in relative cell volume over time upon hypotonic exposure. Data is represented as the mean reciprocal and relative fluorescence intensity (FI) \pm SEM measured by calcein fluorescence self-quenching in mock-transfected (B) and integrin β_1 -siRNA transfected ELA cells (arrow indicates shift to hypotonicity). D. Maximum cell swelling determined after shift to hypotonic environment. E. Maximum recovery rate was given as the maximal 1 minute numeric slope ([FI]/min) after reaching maximal cell swelling. F. Fractional rate constant (min⁻¹) for taurine release was determined from release of ³H-labelled taurine and plotted versus time under isotonic and hypotonic conditions (arrow indicate shift to hypotonicity) in mock-transfected (white) and integrin β_1 -siRNA transfected (black) ELA cells. The number of independent experiments was 6 (panel A to E) and 4–6 (panel F). Statistical analysis was performed with paired Student's *t*-test, where * indicate *P* < 0.05.

Knockdown of integrin β_1 reduces volume recovery in ELA cells, but does not affect taurine efflux

As mentioned in the introduction, the mechanism of Ehrlich cell volume sensing is still unknown. We therefore investigated the role of integrin β_1 in hypotonic cell swelling and RVD in ELA and EATC-WT cells. ELA and EATC-WT cells were transiently transfected with 5 nM negative control (mock) or integrin β_1 siRNA for 24–48 h. Knockdown efficiency was determined with western blot analysis, and integrin β_1 protein level was reduced by 70–90% (Fig. 4A and 5A). Changes in cell volume in mock- and integrin β_1 -siRNA transfected ELA cells were followed as changes in fluorescence intensity mediated by calcein-dilution/up-concentration over time (Fig. 4B and 4C). Mock-transfected and integrin β_1 knockdown ELA cells swelled to the same extent when exposed to a hypotonic environment (Fig. 4B–D). However, the maximum recovery rate is significantly reduced in integrin β_1 knockdown cells compared to mock-transfected cells, i.e., the volume recovery is 0.046 ± 0.004 FI/min in mock-transfected ELA cells and 0.029 ± 0.006 FI/min in integrin β_1 -siRNA transfected ELA

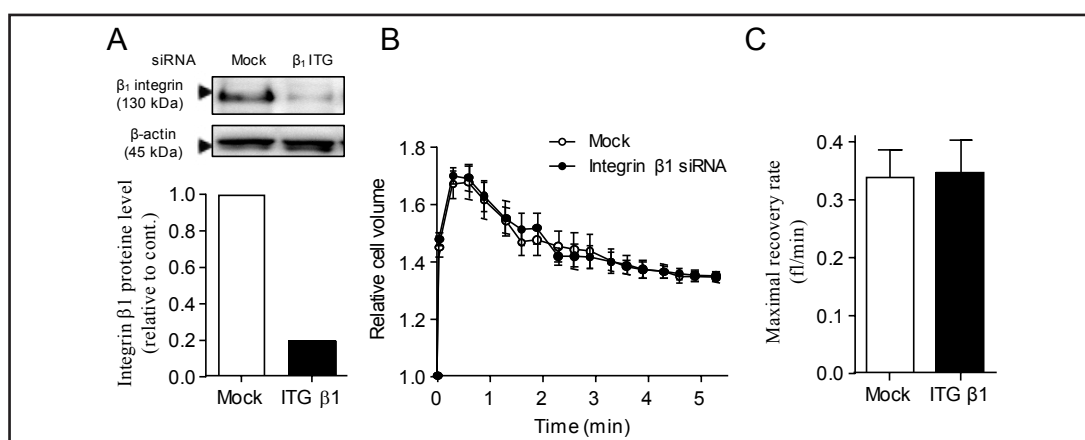
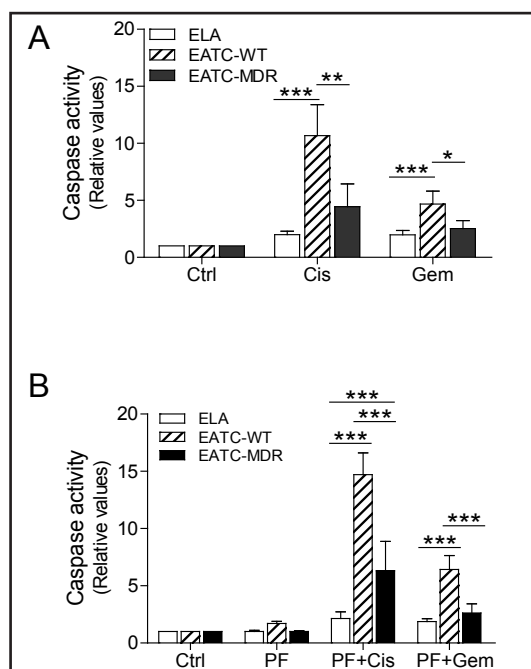


Fig. 5. Effect of siRNA-mediated knockdown of integrin β_1 on cell swelling and RVD in EATC-WT. Changes in cell volume and RVD upon hypotonic exposure in EATC-WT transfected with 5 nM mock- or integrin β_1 -siRNA for 48 h. A. Knockdown efficiency determined in one of the experiments. B. Changes in relative cell volume (fl) \pm SEM over time upon hypotonic exposure measured by electric cell sizing in mock- (open symbols) and integrin β_1 -siRNA transfected (black symbols) EATC-WT cells. C. Maximum recovery rate was given as the maximal 1 minute numeric slope (fl/min) after reaching maximal cell swelling. The number (n) of independent experiments per condition was 3.

Fig. 6. Effect of cisplatin, gemcitabine, and FAK inhibitor PF-573,228 on caspase activity in ELA, EATC-WT, and EATC-MDR cells. ELA, EATC-WT, and EATC-MDR cells were treated with 5 μ M cisplatin or 5 μ g/ml gemcitabine for 24 h in the absence (A) or presence of 10 μ M PF-573,228 (PF)(B), and the cells were subsequently assayed for caspase activity with a fluorimetric caspases assay. Data is shown as mean with SEM error bars, where ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, obtained by two-way ANOVA with Bonferroni multiple comparisons post test. The number (n) of independent experiments per condition was 5-8.



cells (Fig. 4E). This indicates that integrin β_1 contributes to RVD. Cell swelling and RVD were in EATC-WT followed by electric cell sizing using a coulter counter (Fig. 5B). Knockdown of integrin β_1 in EATC-WT had no effect on cell swelling and RVD (Mock, 0.34 ± 0.05 fl/min; Integrin β_1 , 0.35 ± 0.06 fl/min) (Fig. 5B and 5C). These results indicate that integrin β_1 plays an important role in RVD in adherent ELA cells, whereas it has no effect on RVD in non-adherent EATC-WT.

Pharmacological evidence has indicated that release of the organic osmolyte taurine, which is known to contribute to RVD, is regulated by RTKs, FAK, RhoA, and members of the Src family, which are all kinases known to be regulated by integrin clustering and activation [53-56]. We therefore investigated the effect of integrin β_1 knockdown on swelling-induced taurine efflux via the volume sensitive organic anion channel (VSOAC). Figure 4F shows

the fractional rate constant versus time for mock- and integrin β_1 -transfected ELA cells. Integrin β_1 -siRNA transfected ELA cells had a maximum taurine efflux of $0.063 \pm 0.004 \text{ min}^{-1}$ compared to $0.061 \pm 0.007 \text{ min}^{-1}$ in mock-transfected cells, indicating that integrin β_1 is not involved in the activation of VSOAC in ELA cells. Taken together these data suggest that integrin β_1 contributes significantly to RVD in adherent ELA cells in a way independent of VSOAC, whereas integrin β_1 has no effect on cell swelling and RVD in the non-adherent EATC-WT cells.

Non-adherent EATC-WT cells are significantly more sensitive to drug-induced apoptosis than the resistant EATC-MDR and the adherent ELA cells

Previously, ELA and EATC-MDR cells have both been shown to be resistant to cisplatin-induced apoptosis, while EATC-WT readily undergoes apoptosis after exposure to cisplatin [47, 48]. To further elucidate the mechanisms underlying resistance, the sensitivities of the three cell lines to the chemotherapeutic drugs cisplatin and gemcitabine were compared. EATC-WT, EATC-MDR, and ELA cells were treated with $5 \mu\text{M}$ cisplatin or $5 \mu\text{g/ml}$ gemcitabine for 24 h, followed by measurement of the caspase activity.

Both cisplatin and gemcitabine induced significantly higher caspase activation in EATC-WT cells compared to ELA and EATC-MDR cells; cisplatin induced a 10-fold increase in caspase activity in EATC-WT, and only a 4.5-fold increase in EATC-MDR and a 2-fold increase in ELA-cells (Fig. 6), and similarly, treatment with gemcitabine induced a 5-fold increase in caspase activity in EATC-WT cells, a 2.6-fold increase in EATC-MDR cells, and a 2-fold increase in ELA cells (Fig. 6).

The role of FAK in caspase activation was investigated as well, by the use of the FAK inhibitor PF-573,228 (PF). Caspase activity in EATC-MDR and ELA cells was not affected by PF treatment, whereas a 1.7 fold increase was observed in EATC-WT (Fig. 6B). However, exposure of the cells to a combination of cisplatin and PF led to a further increase in caspase activity in EATC-WT and EATC-MDR cells, which was approximately 15-fold and 6-fold, respectively, relative to the untreated control (Fig. 6B). The caspase activity in ELA cells remained unchanged by the combination treatment (Fig. 6B). Simultaneous treatment with gemcitabine and PF also led to a further increase in caspase activity for EATC-WT cells (6.5-fold relative to control), while caspase activity in EATC-MDR and ELA cells remained unchanged relative to treatment with gemcitabine alone (Fig. 6B).

Together, these results indicate that the non-adherent EATC-WT cells are significantly more sensitive to both cisplatin- and gemcitabine-induced apoptosis, and that the adherent wild-type ELA cells appear to be the most resistant. In addition, the FAK inhibitor PF did not affect resistance in the adherent ELA cells, indicating that FAK is not important in either cisplatin or gemcitabine resistance in ELA cells.

Knockdown of integrin β_1 increases the sensitivity of the adherent ELA cells to cisplatin-induced apoptosis

We investigated the role of integrin β_1 knockdown on cisplatin- and gemcitabine-induced apoptosis. EATC-WT, EATC-MDR, and ELA cells were transiently transfected with 5 nM integrin β_1 or negative control (mock) siRNA for 24 h. Subsequently, cells were treated with $5 \mu\text{M}$ cisplatin or $5 \mu\text{g/ml}$ gemcitabine for 24 h. Caspase activity was measured with a fluorimetric caspases assay, and knockdown efficiency was determined with western blot analysis.

The protein levels of mature integrin β_1 in EATC-WT, EATC-MDR, and ELA cells were reduced by about 70%, 80%, and 90%, respectively, compared to mock-transfected cells at 72 h after transfection (Fig. 7A). siRNA transfection did not affect the expression of β -actin in any of the cell lines.

In ELA cells, knockdown of integrin β_1 led to a small increase in caspase activity relative to mock-transfected cells (Fig. 7B). Treatment with cisplatin further increased the caspase activity in both mock-transfected and integrin β_1 -knockdown ELA cells relative to the untreated cells (Fig. 7B). The relative difference in caspase activity between mock-

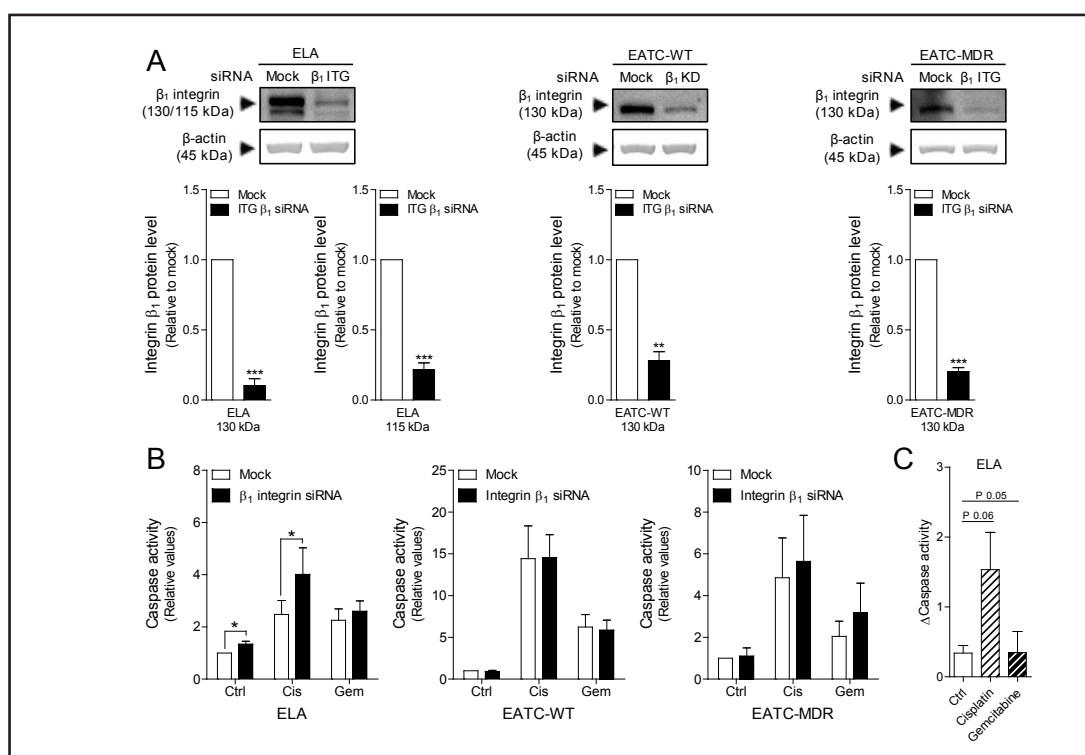


Fig. 7. Effect of siRNA-mediated knockdown of β_1 integrin on chemosensitivity towards cisplatin and gemcitabine in ELA, EATC-WT, and EATC-MDR cells. ELA, EATC-WT, and EATC-MDR cells were transiently transfected with 5 nM mock- or integrin β_1 -siRNA for 24 h. A. Efficiency of siRNA knockdown was determined with western blot analysis, using a specific anti-integrin β_1 antibody. β -actin was used as a loading control. B. After transfection, cells were treated with 5 μ M cisplatin or 5 μ g/ml gemcitabine for 24 h, and cells were subsequently assayed for caspase activity with a fluorimetric caspases assay. Data is shown as mean with SEM error bars normalized to the mock-transfected control. The number (n) of independent experiments was 3-6. Statistical analysis was performed with paired Student's *t*-test, where *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. C. The difference in caspase activity (Δ caspase activity) between mock- and β_1 integrin siRNA-transfected ELA cells.

transfected and integrin β_1 knockdown ELA cells (Δ caspase activity) was 4.5-fold higher ($P = 0.056$) in cells receiving cisplatin treatment compared to untreated cells (Fig. 7C). This is taken to indicate that ELA cells become sensitive to cisplatin treatment following integrin β_1 knockdown. A similar pattern was observed in ELA cells receiving gemcitabine treatment; caspase activation was increased in both mock-transfected and integrin β_1 knockdown cells relative to the untreated cells (Fig. 7B). However, Δ caspase activity in integrin β_1 -knockdown ELA cells was unaffected by gemcitabine treatment (Fig. 7C), indicating that integrin β_1 has no effect on gemcitabine sensitivity in ELA cells.

In EATC-WT and EATC-MDR cells, treatment with either cisplatin or gemcitabine increased caspase activity equally in mock-transfected and integrin β_1 knockdown cells (Fig. 7B), suggesting that knockdown of integrin β_1 does not affect the sensitivity of the non-adherent cells.

From a general point of view, one might suggest that swelling-induced signaling via integrin β_1 /FAK/Src/EGFR leads to cell proliferation as shrinkage-induced signalling leads to caspase activation and apoptosis. To test whether integrin β_1 knockdown also affects proliferation in general we determined cell viability in ELA cells transiently transfected with integrin β_1 siRNA. We find that integrin β_1 knock down does not significantly reduce viability (proliferation) in ELA cells (mock transfected cells: 0.70 ± 0.10 ; integrin β_1 siRNA transfected

cells: 0.57 ± 0.04 , 3 sets of paired experiments). Thus, integrin β_1 knockdown only seems to lead to an increased sensitivity towards chemotherapeutics.

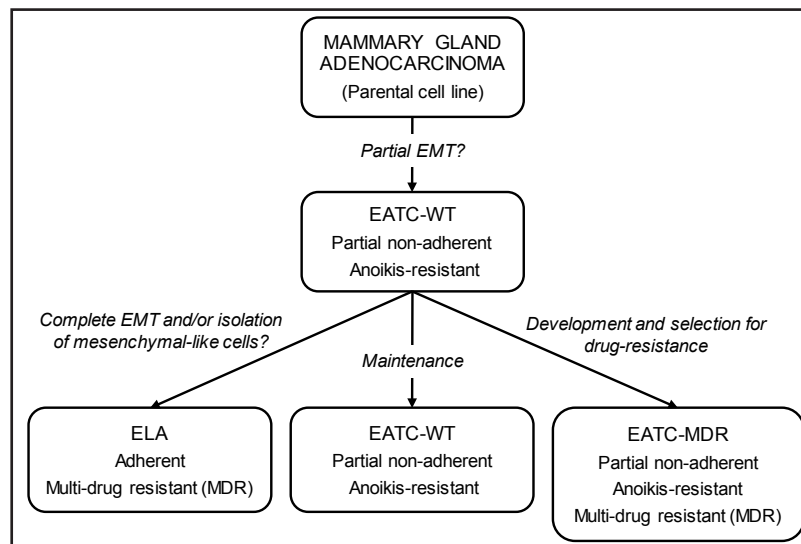
Discussion

Previous studies have identified a linkage between an altered integrin expression, tumor cell progression, metastasis, and chemoresistance [1, 5, 57].

In this study, we find that the three Ehrlich cell lines express equal and relatively high levels of $\alpha_6\beta_1$ and $\alpha_3\beta_1$, whereas integrin α_5 , α_v , and β_1 were found to be higher in adherent ELA cells compared to the two suspension cell lines. Integrin α_2 expression was low in all three cell lines. The integrin-heterodimers expressed by epithelial cells are often retained by tumor cells derived from these [1]. Widely expressed integrin heterodimers found in epithelial cells include $\alpha_6\beta_4$, $\alpha_6\beta_1$, and $\alpha_3\beta_1$. These integrins mediate the attachment to the basal lamina, but might also contribute to proliferation, migration, and survival of the tumor cells [1]. In this study of the Ehrlich cell lines, which originate from mammary gland adenocarcinomas [49, 50], we show that all three cell lines have a relatively high and equal expression of the laminin receptors $\alpha_6\beta_1$ and $\alpha_3\beta_1$, in line with their epithelial origin.

Other integrins are up-regulated in tumor cells compared to the non-transformed parental cell. Especially, the mesenchymal integrins, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_5\beta_1$, are often only expressed at low or even undetectable levels in adult epithelial tissue, but can be highly over-expressed in tumor cells [1]. The process of trans-differentiation of epithelial cells into cells with mesenchymal appearance and properties (epithelial-to-mesenchymal transition; EMT) is a normal process during fetal development, but can also be associated with pathogenesis, such as cancer progression and drug-resistance, as observed in the ELA cells [58]. Various secreted ligands are reported to induce EMT, including GFs (e.g., fibroblast growth factor, transforming growth factor β , and EGF), Wnt, and Hedgehog [58]. RTK trans-activation through GF stimulation results in down-stream activation of Rho GTPases, MAPK pathways, and the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway, which are signaling cascades also known to be important for volume regulation, evasion of anoikis

Fig. 8. Relation and properties of the Ehrlich cell lines. The Ehrlich cells were first described in 1905 by P. Ehrlich, as transplantable tumors arising as spontaneous mammary gland adenocarcinomas in female mice. These cells were in 1930s transformed into anoikis-resistant suspension cells able to grow in the abdomen of mice and are today known as wild-type Ehrlich ascites tumor (EATC-WT) cells. In the



1960s, Hans Lettré grew an adherent version of the Ehrlich cells, today known as Ehrlich Lettré ascites (ELA) tumor cells, and these cells are grown in monolayer. The ELA cells are in addition found to be highly resistant to the chemotherapeutic drug, cisplatin, to which EATC-WT cells are sensitive. Keld Danø developed a special multidrug resistant variant of EATC-WT cells (EATC-MDR) by treating the wild-type cells with the chemotherapeutic drug Daunorubicin over several passages [51].

during cancer progression and metastatic spread, as well as development of drug resistance [58-60]. Petras and colleagues have recently documented a direct correlation between increased ErbB1 expression and a corresponding increase in integrin β_1 expression, as well as Akt-mediated radio-resistance in astrocytomas [61]. In the adherent ELA cells, which are derived from the non-adherent EATC-WT cells (see properties in Fig. 8), integrin α_5 , α_v , and β_1 were found to be over-expressed compared to the two suspension cell lines. Especially, integrin α_5 was highly expressed in ELA cells. Increased expressions of integrin $\alpha_5\beta_1$ and $\alpha_v\beta_1$ have in clinical studies been associated with a decreased survival rate of patients with acute myeloid leukemia, melanomas, and non-small cell lung cancer [62-65]. In addition to the GF-regulated increase in integrin expression, integrins are also reported to potentiate GF signaling through intercalation of signaling pathways and direct GF cross-talk [66-68]. The data indicate that the ELA cells might be a more aggressive cancer cell line than EATC-WT and EATC-MDR and that the EMT transition at some time point might have played an important role in the development of the ELA cell line from EATC (Fig. 8).

In contrast to $\alpha_5\beta_1$ and $\alpha_v\beta_1$, the collagen-binding integrin $\alpha_2\beta_1$ is often highly expressed in normal breast ducts and ductules, but can be markedly reduced or undetectable in poorly differentiated breast adenocarcinomas and other epithelial malignancies [69, 70]. Re-expression of integrin $\alpha_2\beta_1$ in breast carcinoma cells with no detectable $\alpha_2\beta_1$ expression was moreover found to abrogate the malignant phenotype and re-establish cell differentiation into mammary gland-like structures [71]. In our study, we find that all three Ehrlich cell lines exhibit a low expression of integrin $\alpha_2\beta_1$, which could possibly reflect the down-regulation associated with tumorigenesis that has been observed in the mentioned studies.

Increased expression of integrin β_1 contributes to RVD in adherent cells

Our findings illustrate that integrin β_1 regulates RVD in adherent ELA cells but not in the closely related suspension cell line EATC-WT. Integrins have several properties that could identify them as potential volume sensors. As integrins are situated in the plasma membrane and directly links the extracellular matrix to the intracellular milieu (e.g., actin cytoskeleton and intracellular signaling pathways), integrins might be directly subjected to structural changes caused by volume perturbations. Integrin β_1 has already been identified as being the “volume-sensor” and activator of VRAC through EGF receptor trans-activation and down-stream GF-related signaling (e.g., PI3K, Akt, and MAPK) in rat hepatocytes and rabbit ventricular myocytes, respectively [14, 24]. Knockdown of integrin β_1 in adherent ELA cells was found to reduce volume recovery with approximately 40%, thus indicating that integrin β_1 is not the “volume sensor” per se but might contribute to a larger volume sensory network, comprising other integrin heterodimers, e.g., integrin $\alpha_v\beta_3$ or $\alpha_v\beta_6$, as well as other volume-sensory biomolecules, such as GF receptors. Other observations supporting that integrins are not the volume sensor is the fact that volume-sensing was maintained after integrin knock-down in suspension Ehrlich cells.

Several studies have suggested that cross-communication between channels and integrins exist, and that fibronectin binding promotes activation of various channels, including Ca^{2+} -dependent K^+ channels, inwardly rectifying K^+ channels, G-protein gated inwardly rectifying channels (GIRK), the Na^+/K^+ ATPase, voltage gated Ca^{2+} channels (V CaC), the epithelial sodium channel (ENaC), and the human ether-à-go-go-related gene 1 (hERG) K^+ channel currents [72-79]. Regarding channels important for volume regulation, the voltage-gated K^+ channel $\text{K}_v1.3$ [17-19] and $\text{K}_v11.1$ [20] have, as described in the introduction, been found to be physically and functionally linked to β_1 integrins.

RVD in Ehrlich cell lines does not rely on voltage-gated K^+ channels [80, 81]; instead, VRAC, TASK2, potassium-chloride-co-transporter (KCC) 1 and 3, and VSOAC are known to be important [9]. A direct physiological link between integrins and the volume regulated channels found in Ehrlich cells is lacking, but in cardiac ventricular myocytes VRAC has been found to be regulated by integrin β_1 stretch [21-24]. Important components of VRAC and VSOAC, which are by some authors suggested to be identical, have recently been identified as being members of the Leucine-rich repeat containing 8 (LRRC8) family [82, 83]. Especially,

LRRC8A is found to be important, as siRNA-mediated knockdown abolished the swelling-induced Cl^- currents, RVD, and taurine efflux. LRRC8 consists of four transmembrane segments followed by a C-terminal leucine-rich-repeat domain and is proposed to form heterohexameric channels like described for pannexins [84]. Our results indicate that activation of VSOAC occurs independently of integrin β_1 in ELA cells. This is somewhat unexpected but supports our previous assumptions that VRAC and VSOAC might not be completely identical, i.e., they differ in activation/inactivation time-frame, effect of cholesterol depletion, and pharmacological profile as well as their dependence on integrin β_1 [24, 85-89], or at least that the selective channel-pore might be differently regulated. It could be suggested that the observed differences between Cl^- and taurine transport might be a result of variations between the subunit stoichiometry of two or more independent LRRC8-based channels and their different upstream regulators. Alternative volume sensitive pathways not involving integrins are mentioned in Introduction and further discussed in [9, 13].

The adherent ELA cells are highly resistant to chemotherapy

The chemotherapeutic drugs cisplatin and gemcitabine induced caspase activity in all three cell lines. Cisplatin appeared to have a stronger cytotoxic effect on these tumor cells compared to gemcitabine. The effect of both drugs was most pronounced in the non-adherent EATC-WT cells and least pronounced in the adherent ELA cells, indicating that EATC-MDR and ELA cells possess extrinsic and intrinsic resistance against these chemotherapeutics, respectively. In support of these results, Poulsen et al. showed that EATC-MDR cells were resistant to cisplatin-induced apoptosis, as demonstrated by an increased cell viability and decreased caspase 3 activity compared to EATC-WT [48]. ELA was the most resistant cell type, supporting the hypothesis that adhesion might be an important factor in drug resistance, perhaps as a result of a different integrin composition and integrin-mediated survival signals in these cells (e.g., the MAPK and PI3K/Akt/mTOR pathways). Tastesen et al. also observed a higher resistance in ELA cells compared to EATC-WT cells, and found that cisplatin induced caspase 3 activity in EATC-WT but not in ELA cells [47]. Our results also confirm the multidrug resistant nature of EATC-MDR cells, as they exhibited cross-resistance to cisplatin and gemcitabine, which are structurally unrelated and might possess different cytostatic actions compared with daunorubicin, which was used to select drug resistance in these cells.

Treatment with the FAK inhibitor PF potentiated the cytotoxic effect of cisplatin in EATC-WT and EATC-MDR cells as well as gemcitabine in EATC-WT. This suggests that survival signaling in the suspension cells may be partly regulated by FAK, and that disruption of signaling from for example cell-cell contacts [90] might induce apoptosis. Since drug-induced caspase activation in ELA cells was unaffected by the FAK inhibitor, chemoresistance in these cells might not only be regulated by adhesion, as FAK then would be the most obvious signaling mediator [91, 92]. Tastesen et al. suggested three putative mechanisms for the cisplatin resistance of ELA cells, namely lower nuclear accumulation and DNA-binding of cisplatin; increased activity of the taurine transporter, leading to intracellular accumulation of the cytoprotective osmolyte taurine; and decreased anion and water loss, which is a crucial part of apoptotic volume decrease (AVD) [47]. More recently it was demonstrated that acquired cisplatin resistance in human ovarian A2780 cancer cells correlated with an increased taurine accumulation due to increased activity of the taurine accumulating transporter TauT and a concomitant reduction in the volume sensitive taurine release pathway [93].

Knockdown of integrin β_1 reduces cisplatin resistance in adherent cells

Compared to mock-transfected cells, knockdown of integrin β_1 increased the caspase activity in untreated ELA cells. Furthermore, cisplatin-induced caspase activity was increased by knockdown of integrin β_1 , indicating that knockdown of integrin β_1 enhances the cytotoxic effect of cisplatin and thus increases the chemosensitivity of ELA cells to cisplatin. Integrin β_1 knockdown did not affect cell viability (proliferation) significantly in ELA cells. We did

not elucidate the exact mechanism for integrin β_1 -mediated chemoresistance in ELA cells; however, it has been shown that integrin β_1 silencing sensitizes non-small cell lung cancer cells to cisplatin treatment due to a defective activation of the EGFR signaling cascade [94]. Another mechanism mediating cisplatin resistance in ELA cells could involve integrin β_1 -mediated up-regulation of the multidrug resistance-associated protein-1 (MRP-1), as binding of collagen to integrin β_1 has been found to up-regulate expression and activity of MRP-1 in leukemic T-cells [95], and expression of MRP-1 has been correlated to cisplatin resistance of lung cancer cells [96]. Knockdown of integrin β_1 did not affect the sensitivity of ELA cells to gemcitabine, pointing to other resistance mechanisms for this drug. As the exact cellular action of cisplatin and gemcitabine are still incompletely understood it is not possible to give a mechanistic explanation for the difference.

In conclusion, our studies illustrate that the adherent ELA cell line has strong fibronectin binding capacities and a higher expression of the mesenchymal integrin subunits α_5 , α_v , and β_1 compared to the two closely related suspension cell lines (EATC-WT and EATC-MDR), indicating that ELA cells have undergone EMT during development. We also demonstrate that the increased expression of integrin β_1 observed in ELA cells directly improves osmosensing and cisplatin resistance partially by activation of FAK. In contrast, integrin β_1 has no effect on osmosensing and drug resistance in the suspension adapted cell lines EATC-WT or EATC-MDR. Our results hereby indicate a general integrin-dependent mechanism for osmosensing and development of chemoresistance.

Abbreviations

AVD (Apoptotic volume decrease); BSA (Bovine serum albumin); EATC (Ehrlich ascites tumor cells); ECM (Extracellular matrix); EDTA (Ethylenediaminetetraacetic acid); EGFR (Epidermal growth factor receptor); ELA (Ehrlich lettré ascites tumor cells); EMT (Epithelial-to-mesenchymal transition); ENaC (Epithelial sodium channel); ERK1/2 (Extracellular signal-regulated kinase 1 and 2); FAK (Focal adhesion kinase); FGF (Fibroblast growth factor); FRET (Fluorescence resonance energy transfer); GIRK (G-protein gated inwardly rectifying channel); GRGDS (Gly-Arg-Gly-Asp-Ser); GF (Growth factor); hERG (Human ether-à-go-go-related gene); HOG1 (High osmolarity glycerol 1); HRP (Horseradish peroxidase); KCC (Potassium-chloride-co-transporter); LRRC8 (Leucine rich repeat containing 8); MAPK (Mitogen activated protein kinase); MDR (Multidrug resistance); MRP-1 (Multidrug resistance-associated protein-1); mTOR (Mammalian target of rapamycin); PBS (Phosphate buffered saline); PF (PF-573,228); PI3K (Phosphatidylinositol 3-kinase); PI5K (Phosphatidylinositol 5-kinase); PLA₂ (Phospholipase A₂); PLC (Phospholipase C); RGD (Arg-Gly-Asp); ROS (Reactive oxygen species); RTK (Receptor Tyrosine Kinase); RVD (Regulatory volume decrease); RVI (Regulatory volume increase); TASK2 (TWIK-related acid-sensitive K⁺ channel 2); TGF β (Transforming growth factor β); TRP (Transient receptor potential); VCaC (Voltage gated Ca²⁺ channel); VRAC (Volume regulated anion channel); VSOAC (Volume sensitive organic anion channel); WT (Wild type).

Acknowledgements

Birthe J. Hansen and Dorthe Nielsen are acknowledged for excellent technical assistance. We thank COST Action CM1105 for stimulating discussions. The following co-authors' current work addresses differ from the affiliation line: Anders Aspberg (Molecular Skeletal Biology, Department of Clinical Sciences, Lund University, Lund, Sweden), Bjørn Sindballe Broberg (Novo Nordisk A/S, Kalundborg, Denmark), Thomas Kjær Klausen (Dako, Glostrup, Denmark) and Line Jee Hartmann Rasmussen (Clinical Research Centre, Copenhagen University Hospital Hvidovre, Hvidovre, Denmark).

The present work was supported by The Carlsberg Foundation, Brødrene Hartmann Foundation (EKH), "Læge Sofus Carl Emil Friis og Olga Doris Friis's legat" (IHL), "Agnes og Poul Friis's Fond" (IHL), and Augustinus Foundation (EKH).

Disclosure Statement

The authors declare that they have no conflict of interest.

References

- 1 Desgrosellier JS, Cheresh DA: Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 2010;10:9-22.
- 2 Brakebusch C, Fassler R: beta 1 integrin function in vivo: adhesion, migration and more. *Cancer Metastasis Rev* 2005;24:403-411.
- 3 Schwab A, Stock C: Ion channels and transporters in tumour cell migration and invasion. *Philos Trans R Soc Lond B Biol Sci* 2014;369:20130102.
- 4 Barczyk M, Carracedo S, Gullberg D: Integrins. *Cell Tissue Res* 2010;339:269-280.
- 5 Aoudjit F, Vuori K: Integrin signaling in cancer cell survival and chemoresistance. *Chemother Res Pract* 2012;2012:283181.
- 6 Gilmore AP: Anoikis. *Cell Death Differ* 2005;12:S1473-1477.
- 7 Dubois JM, Rouzaire-Dubois B: Roles of cell volume in molecular and cellular biology. *Prog Biophys Mol Biol* 2012;108:93-97.
- 8 Pedersen SF, Hoffmann EK, Novak I: Cell volume regulation in epithelial physiology and cancer. *Front Physiol* 2013;4:233.
- 9 Hoffmann EK, Lambert IH, Pedersen SF: Physiology of cell volume regulation in vertebrates. *Physiol Rev* 2009;89:193-277.
- 10 Lambert IH, Kristensen DM, Holm JB, Mortensen OH: Physiological role of taurine - from organism to organelle. *Acta Physiol (Oxf)* 2015;213:191-212.
- 11 Li S, Ault A, Malone CL, Raitt D, Dean S, Johnston LH, Deschenes RJ, Fassler JS: The yeast histidine protein kinase, Sln1p, mediates phosphotransfer to two response regulators, Ssk1p and Skn7p. *EMBO J* 1998;17:6952-6962.
- 12 Bahn YS, Kojima K, Cox GM, Heitman J: A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of *Cryptococcus neoformans*. *Mol Biol Cell* 2006;17:3122-3135.
- 13 Pedersen SF, Kapus A, Hoffmann EK: Osmosensory mechanisms in cellular and systemic volume regulation. *J Am Soc Nephrol* 2011;22:1587-1597.
- 14 Haussinger D, R. Reinehr, Schliess F: The hepatocyte integrin system and cell volume sensing. *Acta Physiol (Oxf)* 2006;187:249-255.
- 15 Vom DS, Schliess F, Reissmann R, Gorg B, Weiergraber O, Kocalkova M, Dombrowski F, Haussinger D: Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver. *J Biol Chem* 2003;278:27088-27095.
- 16 Reinehr R, Sommerfeld A, Haussinger D: Insulin induces swelling-dependent activation of the epidermal growth factor receptor in rat liver. *J Biol Chem* 2010;285:25904-25912.
- 17 Artym VV, Petty HR: Molecular proximity of Kv1.3 voltage-gated potassium channels and beta(1)-integrins on the plasma membrane of melanoma cells: effects of cell adherence and channel blockers. *J Gen Physiol* 2002;120:29-37.
- 18 Levite M, Cahalon L, Peretz A, Hershkovich R, Sobko A, Ariel A, Desai R, Attali B, Lider O: Extracellular K(+) and opening of voltage-gated potassium channels activate T cell integrin function: physical and functional association between Kv1.3 channels and beta1 integrins. *J Exp Med* 2000;191:1167-1176.
- 19 Cahalan MD, Chandy KG: The functional network of ion channels in T lymphocytes. *Immunol Rev* 2009;231:59-87.
- 20 Cherubini A, Hofmann G, Pillozzi S, Guasti L, Crociani O, Cilia E, Di SP, Degani S, Balzi M, Olivotto M, Wanke E, Becchetti A, Defilippi P, Wymore R, Arcangeli A: Human ether-a-go-go-related gene 1 channels are physically linked to beta1 integrins and modulate adhesion-dependent signaling. *Mol Biol Cell* 2005;16:2972-2983.
- 21 Ren Z, Raucci FJ, Browe DM, Baumgarten CM: Regulation of swelling-activated Cl(-) current by angiotensin II signalling and NADPH oxidase in rabbit ventricle. *Cardiovasc Res* 2008;77:73-80.

- 22 Browe DM, Baumgarten CM: EGFR kinase regulates volume-sensitive chloride current elicited by integrin stretch via PI-3K and NADPH oxidase in ventricular myocytes. *J Gen Physiol* 2006;127:237-251.
- 23 Browe DM, Baumgarten CM: Angiotensin II (AT1) receptors and NADPH oxidase regulate Cl⁻ current elicited by beta1 integrin stretch in rabbit ventricular myocytes. *J Gen Physiol* 2004;124:273-287.
- 24 Browe DM, Baumgarten CM: Stretch of beta 1 integrin activates an outwardly rectifying chloride current via FAK and Src in rabbit ventricular myocytes. *J Gen Physiol* 2003;122:689-702.
- 25 Lambert IH, Klausen TK, Bergdahl A, Hougaard C, Hoffmann EK: ROS activate KCl cotransport in nonadherent Ehrlich ascites cells but K⁺ and Cl⁻ channels in adherent Ehrlich Lettre and NIH3T3 cells. *Am J Physiol Cell Physiol* 2009;297:C198-C206.
- 26 Lambert IH: Activation and inactivation of the volume-sensitive taurine leak pathway in NIH3T3 fibroblasts and Ehrlich Lettre ascites cells. *Am J Physiol Cell Physiol* 2007;293:C390-C400.
- 27 Koberle B, Tomicic MT, Usanova S, Kaina B: Cisplatin resistance: Preclinical findings and clinical implications. *Biochimica et Biophysica Acta-Reviews on Cancer* 2010;1806:172-182.
- 28 Pontiggia O, Sampayo R, Raffo D, Motter A, Xu R, Bissell MJ, Joffe EB, Simian M: The tumor microenvironment modulates tamoxifen resistance in breast cancer: a role for soluble stromal factors and fibronectin through beta1 integrin. *Breast Cancer Res Treat* 2012;133:459-471.
- 29 Aoudjit F, Vuori K: Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* 2001;20:4995-5004.
- 30 Yang XH, Flores LM, Li Q, Zhou P, Xu F, Krop IE, Hemler ME: Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. *Cancer Res* 2010;70:2256-2263.
- 31 Gutierrez C, Schiff R: HER2: biology, detection, and clinical implications. *Arch Pathol Lab Med* 2011;135:55-62.
- 32 Lesniak D, Xu Y, Deschenes J, Lai R, Thoms J, Murray D, Gosh S, Mackey JR, Sabri S, Abdulkarim B: Beta1-integrin circumvents the antiproliferative effects of trastuzumab in human epidermal growth factor receptor-2-positive breast cancer. *Cancer Res* 2009;69:8620-8628.
- 33 Zhang CJ, Shen ZJ, Pan CW, Zhong S, Li T, Zhang MG: Engagement of integrin beta1 induces resistance of bladder cancer cells to mitomycin-C. *Urology* 2012;79:638-643.
- 34 Thomas F, Holly JM, Persad R, Bahl A, Perks CM: Fibronectin confers survival against chemotherapeutic agents but not against radiotherapy in DU145 prostate cancer cells: involvement of the insulin like growth factor-1 receptor. *Prostate* 2010;70:856-865.
- 35 Bhatia V, Mula RV, Weigel NL, Falzon M: Parathyroid hormone-related protein regulates cell survival pathways via integrin alpha6beta4-mediated activation of phosphatidylinositol 3-kinase/Akt signaling. *Mol Cancer Res* 2009;7:1119-1131.
- 36 Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, Chilvers ER, Dransfield I, Donnelly SC, Strieter R, Haslett C: Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. *Nat Med* 1999;5:662-668.
- 37 Hodgkinson PS, Elliott T, Wong WS, Rintoul RC, MacKinnon AC, Haslett C, Sethi T: ECM overrides DNA damage-induced cell cycle arrest and apoptosis in small-cell lung cancer cells through beta1 integrin-dependent activation of PI3-kinase. *Cell Death Differ* 2006;13:1776-1788.
- 38 Ju L, Zhou C, Li W, Yan L: Integrin beta1 over-expression associates with resistance to tyrosine kinase inhibitor gefitinib in non-small cell lung cancer. *J Cell Biochem* 2010;111:1565-1574.
- 39 Miyamoto H, Murakami T, Tsuchida K, Sugino H, Miyake H, Tashiro S: Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins. *Pancreas* 2004;28:38-44.
- 40 Armstrong T, Packham G, Murphy LB, Bateman AC, Conti JA, Fine DR, Johnson CD, Benyon RC, Iredale JP: Type I collagen promotes the malignant phenotype of pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2004;10:7427-7437.
- 41 Hehlhans S, Haase M, Cordes N: Signalling via integrins: implications for cell survival and anticancer strategies. *Biochim Biophys Acta* 2007;1775:163-180.
- 42 Shain KH, Dalton WS: Environmental-mediated drug resistance: a target for multiple myeloma therapy. *Expert Rev Hematol* 2009;2:649-662.
- 43 Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D, Park C: Increased beta1 integrin is associated with decreased survival in invasive breast cancer. *Cancer Res* 2007;67:659-664.

- 44 Oshita F, Kameda Y, Ikehara M, Tanaka G, Yamada K, Nomura I, Noda K, Shotts A, Fujita A, H. Arai H, Ito H, Nakayama H, Mitsuda A: Increased expression of integrin beta1 is a poor prognostic factor in small-cell lung cancer. *Anticancer Res* 2002;22:1065-1070.
- 45 Park CC, Zhang HJ, Yao ES, Park CJ, Bissell MJ: Beta1 integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. *Cancer Res* 2008;68:4398-4405.
- 46 Nielsen K: A hexaploid Ehrlich-Lettre mouse ascites tumor line with low sensitivity to colchicine. *Hereditas* 1976;83:105-122.
- 47 Tastesen HS, Holm JB, Moller J, Poulsen KA, Moller C, Sturup S, Hoffmann EK, Lambert IH: Pinpointing differences in cisplatin-induced apoptosis in adherent and non-adherent cancer cells. *Cell Physiol Biochem* 2010;26:809-820.
- 48 Poulsen KA, Andersen EC, Hansen CF, Klausen TK, Hougaard C, Lambert IH, Hoffmann EK: Deregulation of apoptotic volume decrease and ionic movements in multidrug-resistant tumor cells: role of chloride channels. *Am J Physiol Cell Physiol* 2010;298:C14-C25.
- 49 Bertalanffy FD, Schahter R, Ali J, Ingmundson JC: Mitotic rate and doubling time of intraperitoneal and subcutaneous Ehrlich ascites tumor cells. *Cancer Res* 1965;25:685-691.
- 50 Ozaslan M, Karagoz ID, Kilic IH, Guldur ME: Ehrlich ascites carcinoma. *Afr J Biotechnol* 2011;10:2375-2378.
- 51 Dano K: Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochim Biophys Acta* 1973;323:466-483.
- 52 Bellis SL, Newman E, Friedman EA: Steps in integrin beta1-chain glycosylation mediated by TGFbeta1 signaling through Ras. *J Cell Physiol* 1999;181:33-44.
- 53 Lambert IH: Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. *J Membr Biol* 2003;192:19-32.
- 54 Hubert EM, Musch MW, Goldstein L: Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. *Pflugers Arch* 2000;440:132-139.
- 55 Franco R, Lezama R, Ordaz B, Pasantes-Morales H: Epidermal growth factor receptor is activated by hyposmolarity and is an early signal modulating osmolyte efflux pathways in Swiss 3T3 fibroblasts. *Pflugers Arch* 2004;447:830-839.
- 56 Pedersen SF, Beisner KH, Hougaard C, Willumsen BM, Lambert IH, Hoffmann EK: Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. *J Physiol* 2002;541:779-796.
- 57 Sun CC, Qu XJ, Gao ZH: Integrins: players in cancer progression and targets in cancer therapy. *Anticancer Drugs* 2014; doi: 10.1097/CAD.0000000000000145.
- 58 Derynck R, Muthusamy BP, Saeteurn KY: Signaling pathway cooperation in TGF-beta-induced epithelial-mesenchymal transition. *Curr Opin Cell Biol* 2014;31C:56-66.
- 59 Takeuchi K, Ito F: Receptor tyrosine kinases and targeted cancer therapeutics. *Biol Pharm Bull* 2011;34:1774-1780.
- 60 Martin SS, Vuori K: Regulation of Bcl-2 proteins during anoikis and amorphosis. *Biochim Biophys Acta* 2004;1692:145-157.
- 61 Petras M, Lajtos T, Friedlander E, Klekner A, Pintye E, Feuerstein BG, Szollosi J, Vereb G: Molecular interactions of ErbB1 (EGFR) and integrin-beta1 in astrocytoma frozen sections predict clinical outcome and correlate with Akt-mediated in vitro radioresistance. *Neuro Oncol* 2013;15:1027-1040.
- 62 Adachi M, Taki T, Higashiyama M, Kohno N, Inufusa H, Miyake M: Significance of integrin alpha5 gene expression as a prognostic factor in node-negative non-small cell lung cancer. *Clin Cancer Res* 2000;6:96-101.
- 63 Danen EH, Berge PJT, Van Muijen GN, Van 't Hof-Grootenboer AE, Brocker EB, Ruiter DJ: Emergence of alpha 5 beta 1 fibronectin- and alpha v beta 3 vitronectin-receptor expression in melanocytic tumour progression. *Histopathology* 1994;24:249-256.
- 64 Nikkola J, Vihinen P, Vlaykova T, Hahka-Kemppinen M, Heino J, Pyrhonen S: Integrin chains beta1 and alphav as prognostic factors in human metastatic melanoma. *Melanoma Res* 2004;14:29-37.
- 65 Walter RB, Laszlo GS, Alonzo TA, Gerbing RB, Levy S, Fitzgibbon MP, Gudgeon CJ, Ries RE, Harrington KH, Raimondi SC, Hirsch BA, Gamis AS, McIntosh W, Meshinchi S: Significance of expression of ITGA5 and its splice variants in acute myeloid leukemia: a report from the Children's Oncology Group. *Am J Hematol* 2013;88:694-702.

- 66 Schneller M, Vuori K, Ruoslahti E: Alphavbeta3 integrin associates with activated insulin and PDGFbeta receptors and potentiates the biological activity of PDGF. *EMBO J* 1997;16:5600-5607.
- 67 Fujita M, Takada YK, Takada Y: Insulin-like growth factor (IGF) signaling requires alphavbeta3-IGF1-IGF type 1 receptor (IGF1R) ternary complex formation in anchorage independence, and the complex formation does not require IGF1R and Src activation. *J Biol Chem* 2013;288:3059-3069.
- 68 Yoon SO, Shin S, Lipscomb EA: A novel mechanism for integrin-mediated ras activation in breast carcinoma cells: the alpha6beta4 integrin regulates ErbB2 translation and transactivates epidermal growth factor receptor/ErbB2 signaling. *Cancer Res* 2006;66:2732-2739.
- 69 Ramirez NE, Zhang Z, Madamanchi A, Boyd KL, O'Rear LD, Nashabi A, Li Z, Dupont WD, Zijlstra A, Zutter MM: The alpha(2)beta(1) integrin is a metastasis suppressor in mouse models and human cancer. *J Clin Invest* 2011;121:226-237.
- 70 Albelda SM: Role of integrins and other cell adhesion molecules in tumor progression and metastasis. *Lab Invest* 1993;68:4-17.
- 71 Zutter MM, Santoro SA, Staatz WD, Tsung YL: Re-expression of the alpha 2 beta 1 integrin abrogates the malignant phenotype of breast carcinoma cells. *Proc Natl Acad Sci U S A* 1995;92:7411-7415.
- 72 Shakibaei M, Mobasher A: Beta1-integrins co-localize with Na, K-ATPase, epithelial sodium channels (ENaC) and voltage activated calcium channels (VACC) in mechanoreceptor complexes of mouse limb-bud chondrocytes. *Histol Histopathol* 2003;18:343-351.
- 73 Arcangeli A, Riccarda BMD, Poli R, Ricupero L, Olivetto M: Mutual contact of murine erythroleukemia cells activates depolarizing cation channels, whereas contact with extracellular substrata activates hyperpolarizing Ca2+-dependent K+ channels. *J Cell Physiol* 1989;139:1-8.
- 74 Arcangeli A, Becchetti A, Bene MRD, Wanke E, Olivetto M: Fibronectin-integrin binding promotes hyperpolarization of murine erythroleukemia cells. *Biochem Biophys Res Commun* 1991;177:1266-1272.
- 75 Becchetti A, Arcangeli A, Bene MRD, Olivetto M, Wanke E: Response to fibronectin-integrin interaction in leukaemia cells: delayed enhancing of a K+ current. *Proc Biol Sci* 1992;248:235-240.
- 76 Hofmann G, Bernabei PA, Crociani O, Cherubini A, Guasti L, Pillozzi S, Lastraoli E, Polvani S, Bartolozzi B, Solazzo V, Gragnani L, Defilippi P, Rosati B, Wanke E, Olivetto M, Arcangeli A: HERG K+ channels activation during beta(1) integrin-mediated adhesion to fibronectin induces an up-regulation of alpha(v)beta(3) integrin in the preosteoclastic leukemia cell line FLG 29.1. *J Biol Chem* 2001;276:4923-4931.
- 77 Cherubini A, Hofmann G, Pillozzi S, Guasti L, Crociani O, Cilia E, Di SP, Degani S, Balzi M, Olivetto M, Wanke E, Becchetti A, Defilippi P, Wymore R, Arcangeli A: Human ether-a-go-go-related gene 1 channels are physically linked to beta1 integrins and modulate adhesion-dependent signaling. *Mol Biol Cell* 2005;16:2972-2983.
- 78 McPhee JC, Dang YL, Davidson N, Lester HA: Evidence for a functional interaction between integrins and G protein-activated inward rectifier K+ channels. *J Biol Chem* 1998;273:34696-34702.
- 79 Arcangeli A, Becchetti A: Integrin structure and functional relation with ion channels. *Adv Exp Med Biol* 2010;674:1-7.
- 80 Christensen O, Hoffmann EK: Cell swelling activates K+ and Cl- channels as well as nonselective, stretch-activated cation channels in Ehrlich ascites tumor cells. *J Membr Biol* 1992;129:13-36.
- 81 Niemeyer MI, Hougaard C, Hoffmann EK, Jorgensen F, Stutzin A, Sepulveda FV: Characterisation of a cell swelling-activated K+-selective conductance of ehrlich mouse ascites tumour cells. *J Physiol* 2000;524:757-767.
- 82 Voss FK, Ullrich F, Munch J, Lazarow K, Lutter D, Mah N, Andrade-Navarro MA, von Kries JP, Stauber T, Jentsch TJ: Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. *Science* 2014;344:634-638.
- 83 Qiu Z, Dubin AE, Mathur J, Tu B, Reddy K, Miraglia LJ, Reinhardt J, Orth AP, Patapoutian A: SWELL1, a plasma membrane protein, is an essential component of volume-regulated anion channel. *Cell* 2014;157:447-458.
- 84 Abascal F, Zardoya R: LRRC8 proteins share a common ancestor with pannexins, and may form hexameric channels involved in cell-cell communication. *Bioessays* 2012;34:551-560.
- 85 Klausen TK, Hougaard C, Hoffmann EK, Pedersen SF: Cholesterol modulates the volume-regulated anion current in Ehrlich-Lettre ascites cells via effects on Rho and F-actin. *Am J Physiol Cell Physiol* 2006;291:C757-C771.

- 86 Lambert IH, Hoffmann EK: Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. *J Membr Biol* 1994;142:289-298.
- 87 Lambert IH: Effect of Arachidonic-Acid, Fatty-Acids, Prostaglandins, and Leukotrienes on Volume Regulation in Ehrlich Ascites Tumor-Cells. *J Membr Biol* 1987;98:207-221.
- 88 Lambert IH, Hoffmann EK: The Role of Phospholipase-A2, and 5-Lipoxygenase in the Activation of K and Cl Channels and the Taurine Leak Pathway in Ehrlich Ascites Tumor-Cells. *Acta Physiol Scand* 1991;143:A33.
- 89 Villumsen KR, Duelund L, Lambert IH: Acute cholesterol depletion leads to net loss of the organic osmolyte taurine in Ehrlich Lettre tumor cells. *Amino Acids* 2010;39:1521-1536.
- 90 Schaller MD: Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci* 2010;123:1007-1013.
- 91 Zhao J, Guan JL: Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* 2009;28:35-49.
- 92 Mitra SK, Hanson DA, Schlaepfer DD: Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* 2005;6:56-68.
- 93 Sorensen BH, Thorsteinsdottir UA, Lambert IH: Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate. *Am J Physiol Cell Physiol* 2014;307:C1071-C1080.
- 94 Morello V, Cabodi S, Sigismund S, Camacho-Leal MP, Repetto D, Volante M, Papotti M, Turco E, Defilippi P: beta1 integrin controls EGFR signaling and tumorigenic properties of lung cancer cells. *Oncogene* 2011;30:4087-4096.
- 95 Azreq MAE, Naci D, Aoudjit F: Collagen/beta1 integrin signaling up-regulates the ABCC1/MRP-1 transporter in an ERK/MAPK-dependent manner. *Mol Biol Cell* 2012;23:3473-3484.
- 96 Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH: Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. *Clin Cancer Res* 2001;7:1798-1804.